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Brief Communication

Immunogenicity of Newcastle disease virus vectors expressing Norwalk virus capsid protein in the presence or absence of VP2 protein

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ABSTRACT

Noroviruses are the most common cause of acute gastroenteritis in humans. Development of an effective vaccine is required for reducing their outbreaks. In order to develop a GI norovirus vaccine, Newcastle disease virus vectors, rLaSota and modified rBC, were used to express VP1 protein of Norwalk virus. Co-expression of VP1 and VP2 proteins by Newcastle disease virus vectors resulted in enhanced expression of Norwalk virus VP1 protein and self-assembly of VP1 protein into virus-like particles. Furthermore, the Norwalk virus-specific IgG response induced in mice by Newcastle disease virus vectors was similar to that induced by baculovirus-expressed virus-like particles in mice. However, the modified rBC vector in the presence of VP2 protein induced significantly higher levels of cellular and mucosal immune responses than those induced by baculovirus-expressed VLPs. These results indicate that Newcastle disease virus has great potential for developing a live Norwalk virus vaccine by inducing humoral, cellular and mucosal immune responses in humans.

Introduction

Human noroviruses are the most frequent cause of viral gastroenteritis in people of all ages (Scallan et al., 2011). Noroviruses are members of the family *Caliciviridae* (Green, 2013). Genus *Norovirus* is divided into 6 genogroups (GI–GVI). The GI and GII genogroups are the most important for human infection. Their genome consists of a linear, positive-sense, single-stranded RNA molecule of 7.6 kb with a 5′ untranslated region (UTR), three open reading frames (ORFs), a 3′UTR, and a poly(A) tail. ORF1 encodes a large nonstructural polyprotein. ORF2 and ORF3 encode structural proteins, the major capsid protein (VP1) and a minor capsid protein (VP2), respectively. VP1 protein is the major immunogenic protein of noroviruses (Ball et al., 1998). Expression of VP1 alone has been shown to produce self-assembled norovirus-like particles (VLPs) that are morphologically and antigenically similar to native virions (Jiang et al., 1992).

There is a need to develop an effective vaccine against norovirus infection, but the inability of noroviruses to grow in cell culture systems has hindered the development of effective vaccines. Only recently, a cell culture system was successfully developed by infecting a human norovirus in human B cells (Jones et al., 2014). To circumvent this obstacle, VLPs produced by the baculovirus expression system has been used as norovirus vaccine candidates. Norwalk virus (NV, GI) VLPs have shown to be immunogenic when delivered through intranasal, oral, or parenteral route in mice (El-Kamary et al., 2010; Estes et al., 2000; Guerrero et al., 2001; Harrington et al., 2002; Lindesmith et al., 2005). A NV VLP vaccine was further evaluated in a phase II human trial (Atmar et al., 2011). Two doses of this VLP candidate vaccine reduced the rate of symptomatic infection by 47% and the overall rate of infection by 26%. Serum blockade antibody titers above 200 were associated with a 72% reduction in frequency of illness and a 57% reduction in infection, providing evidence that pre-challenge blockade antibody titers correlated to protection following vaccination and challenge in human volunteers. However, cross-challenge studies suggest that a multivalent GI and GII vaccine platform is required for broad protection (LoBue et al., 2006). Intramuscular immunization of a bivalent formulation including GII and consensus VLPs induced higher antibody levels than the intranasal route of immunization (Parra et al., 2012). In addition, large-scale manufacture of baculovirus VLP vaccines has not been cost-effective and the protective efficacy of baculovirus VLP vaccines against a broad range of norovirus genogroups and genotypes needs to be improved. For effective delivery and large-scale manufacture of VLPs, alternative expression and delivery systems, such as Venezuelan equine encephalitis and vesicular stomatitis viruses have been evaluated (Baric et al., 2002; Guo et al., 2008; Ma and Li, 2011). However, safety concerns regarding systemic spread causing viremia and potential neurovirulence are associated with these viruses (Bukreyev and Collins, 2008). Therefore, there is a great need to evaluate additional viral vectors for an effective norovirus vaccine.

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Newcastle disease virus (NDV) belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. The genome of NDV is a single-stranded, negative-sense RNA (Samal, 2011). NDV isolates vary greatly in their pathogenicity for chickens, and are categorized into three main pathotypes: lentogenic (avirulent), mesogenic (moderately virulent), and velogenic (highly virulent) (Alexander, 1989). Recombinant lentogenic and mesogenic NDV strains have been evaluated as vaccine vectors for animal and human pathogens (Bukreyev and Collins, 2008). Recently, we have evaluated recombinant NDV (rNDV) as a vaccine vector for norovirus (Kim et al., 2014). rNDV expressing the VP1 protein of genogroup (G) II genotype 4 strain elicited norovirus-specific humoral, mucosal, and cellular immune responses in mice, indicating that NDV has the potential to be used as a live viral vaccine against norovirus infection.

The rapid evolution of norovirus genotypes and changing of glycan specificities provide new challenges to norovirus vaccine trials (Ramani et al., 2014). Cross-challenge studies suggest that a multivalent GI and GII vaccine platform is required for broad protection (LoBue et al., 2006). Although our previous study showed expression of VP1 protein of norovirus genotype II.4 strain using rNDV vectors, it will be necessary to formulate NDV vectored vaccines expressing VP1 proteins of multiple genotypes for broad protection using a prime-boost approach. Therefore, in this study, we have evaluated whether rNDV can also be used as a vaccine vector for genogroup I (GI) viruses, which are the most common cause of water-borne norovirus outbreaks (Matthews et al., 2012). VP1 protein of the prototype strain, NV was expressed by two different rNDV vectors, since expression of VP1 protein and induction of immune response in mice can be affected by the type of rNDV vector (Kim et al., 2014).

Results

Generation of rNDVs expressing NV VP1 protein

The ORF2 (1593 nt) of norovirus strain Norwalk virus (Hu/NV/Norwalk virus/1968/US) (Fernandez-Vega et al., 2004) was inserted into cDNAs encoding the complete antigenomic RNAs of NDV strain rLaSota (conventional vector) and a modified rNDV for comparison of the expression levels of VP1 protein (Fig. 1). NDV strain tLaSota is an avirulent lentogenic virus that has been widely used as a vector for vaccine development. The modified vector contained the cDNA of the mesogenic NDV strain Beaudette C (BC) in which the sequence of the F protein cleavage site and HN protein was modified to those of lentogenic strain LaSota (rBCm). rNDVs were readily recovered using our standard procedure (Huang et al., 2001). The correct sequence of the NV insert in each recovered virus was confirmed by RT-PCR and sequence analysis.

Expression of the VP1 protein by rNDV vectors was evaluated in DF1 cells infected with rLaSota-NV ORF2, rBCm-NV ORF2, or their

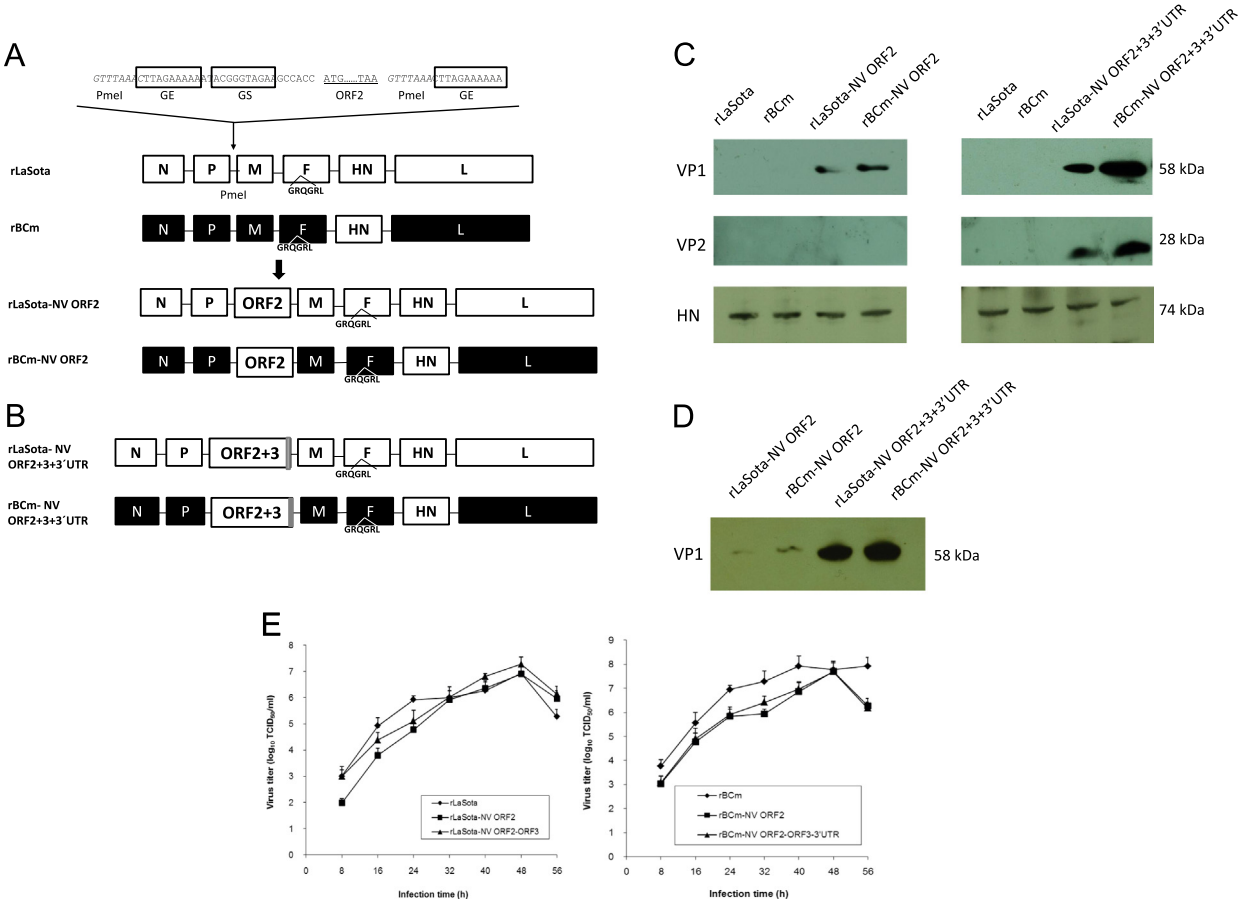

Fig. 1. Generation of rNDVs containing NV ORF2 and production of VP1 protein by rNDVs and in vitro multicycle growth of parental and vaccine viruses in DF1 cells. NV ORF2 alone (A) and ORF2, ORF3, and 3′UTR together (B) were flanked by the gene-start and gene-end signals of NDVs and inserted into the intergenic region between the P and M genes in a full-length antigenomic cDNA of NDVs. (C) DF1 cells were infected with each virus at MOI 1, and cell lysates were collected at 24 h post-infection for Western blot analysis. VP1 (58 kDa) and VP2 (28 kDa) proteins were detected using VP1 specific monoclonal antibody and VP2 specific antipeptide antiserum, respectively. NDV HN protein was detected using a monoclonal antibody. Each figure represents three independent experiments. (D) Detection of NV VP1 in allantoic fluid of chicken embryonated eggs. Each allantoic fluid was harvested at 72 h post-infection and clarified by centrifugation at 3000 rpm for 10 min. This figure represents three independent experiments. (E) DF1 cells were infected with parental or each vaccine virus at an MOI of 0.01. Exogenous protease was provided in the infected cells. The viral titers were determined by limiting dilution on DF1 cells. Results are represented as mean ± SD for the mean of two independent experiments.

parental viruses by Western blot using a monoclonal antibody to VP1 (Parra et al., 2013). rLaSota-NV ORF2 and rBCm-NV ORF2 expressed relatively low levels of VP1 protein (58 kDa) in DF1 cells (Fig. 1C). This indicates that modification in the transcription unit of VP1 ORF2 would be necessary to enhance its expression levels, since the level of VP1 expression can greatly affect the immunogenicity in vivo (Kim et al., 2014). Previously, the expression of VP1 protein by baculovirus in insect cells was enhanced by the presence of the ORF3 and NV 3′UTR (Bertolotti-Ciarlet et al., 2003). However, the contribution of minor structural protein VP2 to expression level of VP1 protein and immune response of norovirus VLP is not well understood. Therefore, we have further evaluated whether VP2 protein can affect expression of VP1 protein and immune response in mice. A transcription cassette containing ORF2, ORF3, and 3′UTR (ORF2+3+3′UTR, 2297 nt) was placed into cDNAs of rLaSota and rBCm vectors (Fig. 1B). Western blot analysis confirmed the expression of the VP2 protein by rNDV vectors and showed enhanced expression of VP1 protein in the presence of ORF3 and 3′UTR (Fig. 1C). Specifically, rBCm expressed higher levels of VP1 protein than rLaSota (Lanes 7 and 8). We then compared the expression levels of VP1 protein by rNDVs in the allantoic fluid of embryonated chicken eggs (Fig. 1D). Similarly, both rNDV vectors containing ORF2+3+3′UTR produced higher levels of VP1 protein in allantoic fluid than those with only ORF2.

In vitro characterization and attenuation of rNDVs expressing VP1 protein

Growth kinetics of parental and rNDVs expressing VP1 protein in DF1 cells was determined to evaluate the effect of insertion of different combination of foreign genes in the NDV genome on virus replication (Fig. 1E). DF1 cells in the presence of 10% chicken egg allantoic fluid were infected with each rNDV-VP1 at an MOI of 0.01. Virus titers in the collected supernatants at 12-h intervals were quantified in DF1 cells by limiting dilution. All the viruses were able to replicate efficiently in the DF1 cells. In general, all of the rNDVs-NV ORF2 and rNDVs-NV ORF2+3+3′UTR viruses replicated less efficiently than their parental viruses at 24 h post-infection (hpi). However, they replicated to high titers at 48 hpi.

Pathogenicity of rNDVs expressing NV VP1 protein was determined by the mean death time (MDT) in 9-day-old SPF embryonated chicken eggs and by the intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (Alexander, 1989). MDT values for each virus were 117 h (rLaSota), 135 h (rLaSota-NV ORF2), 138 h (rLaSota-NV ORF2+3+3′UTR), 108 h (rBCm), 125 h (rBCm-NV ORF2), and 132 h (rBCm-NV ORF2+3+3′UTR). All rNDVs expressing VP1 protein were more attenuated than their parental viruses. Thus, introduction of VP1 protein to rNDV genome conferred virus attenuation. In addition, the ICPI values of all rNDVs were 0.00, and chicks infected with rNDVs had no apparent clinical signs during the 8-day period of the ICPI test. This result suggests that rNDVs expressing the VP1 protein are avirulent in chickens.

Production of NV VLPs by rLaSota and rBCm

We examined whether the NV VP1 protein produced by rNDV vectors can self-assembled into VLPs (Fig. 2). Kinetics analysis of VP1 expression in DF1 cells infected with rLaSota-NV ORF2+3+3′UTR and rBCm-NV ORF2+3+3′UTR showed that both rNDVs expressed high levels of VP1 protein in cell culture medium at 48 hpi, indicating gradual secretion of the VP1 protein into cell culture medium (Fig. 2A). Since NDV grows to high titer in embryonated eggs, the VP1 protein can also be expressed at high level in allantoic fluid, leading to efficient production of VLPs. The two rNDVs containing ORF2+3+3′UTR grew to high titers ($>10^8$ pfu/ml) in embryonated chicken eggs at 3 dpi (data not shown). Subsequently, kinetic analysis confirmed detection of the highest levels of VP1 protein in allantoic fluids at 3 dpi (Fig. 2A). We further verified the absence of VP1 protein

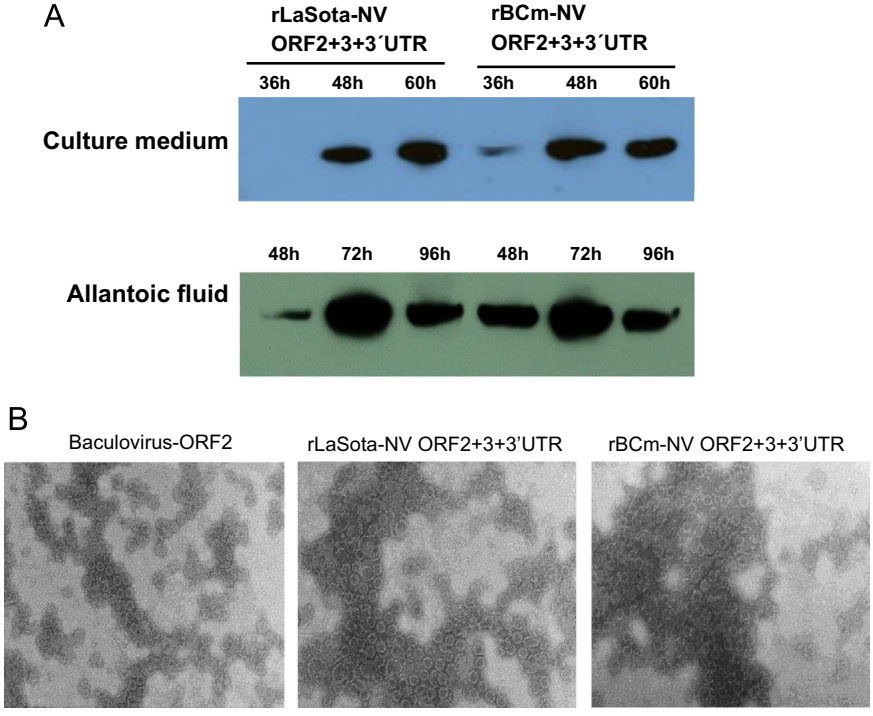

Fig. 2. Characterization of VP1 protein expression and VLP production by rNDVs in DF1 cells and embryonated eggs. (A) Kinetic analysis of VP1 expression by rNDVs was conducted in DF1 cells and in allantoic fluid of chicken embryonated eggs. Cell culture medium was collected every 12 h and subjected to Western blot analysis. Allantoic fluid was harvested every 24 h post-infection and clarified by centrifugation at 3000 rpm for 10 min for Western blot analysis. Each figure represents three independent experiments. (B) Production of VLPs by rNDVs was analyzed by electron microscope. VLP suspension (10 μ each) was fixed in copper grids, negatively stained with 1% ammonium molybdate, and visualized by using an electron microscope. Baculovirus-expressed VLP in Sf9 cells was included as a control. VLPs expressed by rNDVs were prepared from infected allantoic fluid of chicken embryonated eggs. Each figure represents five independent experiments.

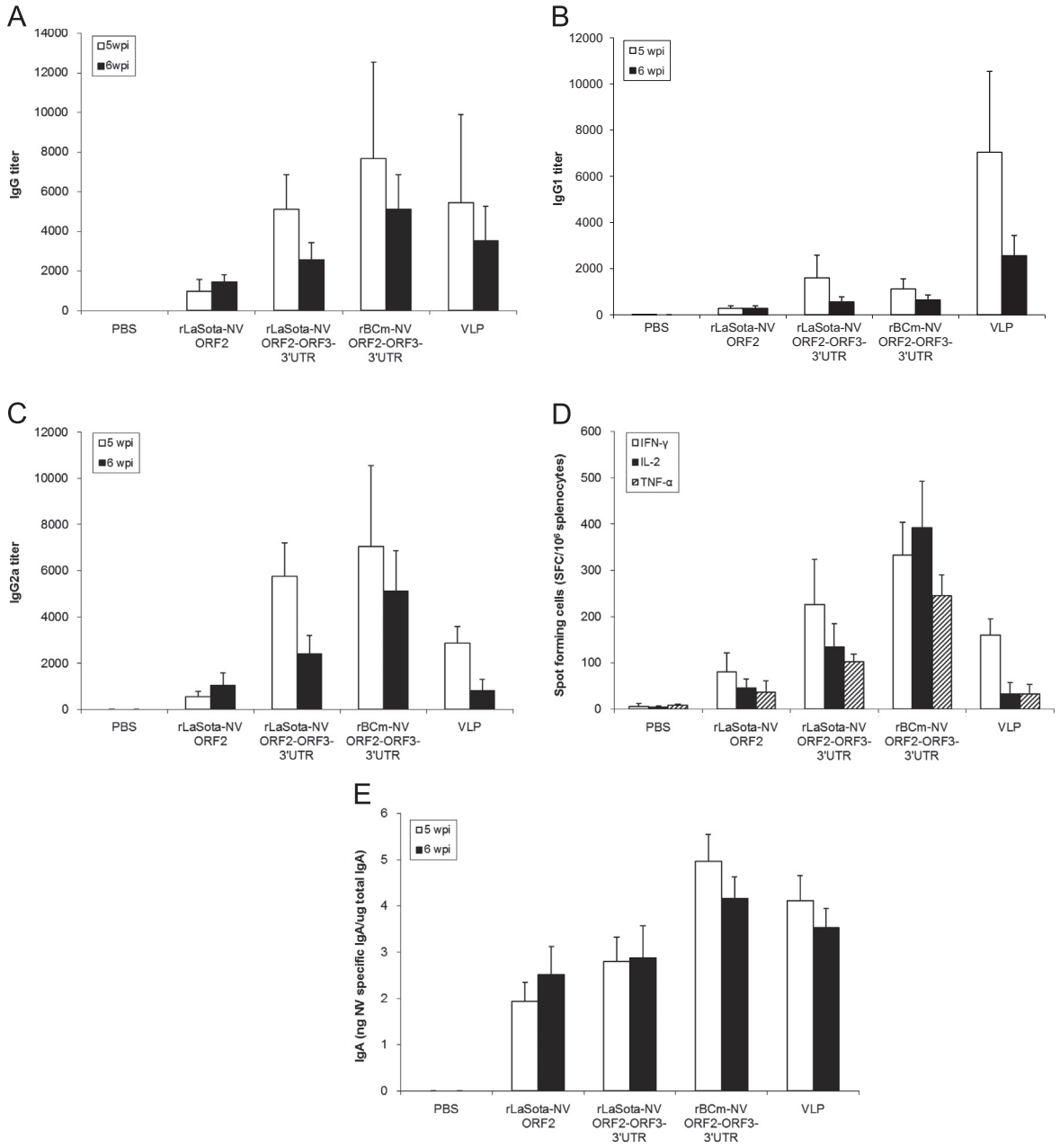

Fig. 3. Antibody titers and NV-specific cellular and fecal IgA responses in mice after immunization with rNDVs and baculovirus-expressed VLPs. Mice were inoculated with each virus and VLPs by the intranasal route for three times in a two-week interval. (A) HBGA binding blocking assay was conducted to evaluate antibody-mediated neutralization for NV. Geometric mean titers (GMTs) for the values of the 50% blocking titer (BT50) determined by the H type 1 blocking assay. The titers of NV-specific total IgG (B) and subtypes IgG1 (C) and IgG2a (D) were determined by ELISA against purified baculovirus-expressed VLPs. The antibody titers were defined as the endpoint dilution with a cut off signal intensity of 0.2. (E) Splenocytes from the immunized mice were stimulated with NV VLP and analyzed for the production of IFN-γ, TNF-α, and IL-2 by the ELISPOT assay. The mean spot-forming cells (SFC)/10^6 cells with the error bars are shown. (F) Fecal samples were diluted in PBS, vortexed, and clarified by centrifugation. NV-specific and total IgA antibodies were determined by ELISA. The ratio between NV-specific IgA and total IgA was determined. Results are represented as mean ± SD for the mean of duplicate samples collected from 5 mice for each group. Statistical significance was determined by ANOVA ($^*P < 0.05$).

in sucrose purified rNDV particles by Western blot (not shown), indicating efficient secretion of VP1 protein into allantoic fluid.

EM analysis was further conducted to evaluate assembly and morphology of VLPs produced by rLaSota-NV ORF2+3+3′UTR and rBCm-NV ORF2+3+3′UTR in allantoic fluid of embryonated chicken eggs compared with VLPs produced in insect cells by baculovirus. For purification of VLPs, allantoic fluid of infected embryonated chicken eggs was collected at 3 dpi and centrifuged at 3000 rpm for 10 min. The VLPs were purified by ultracentrifugation through a 40% sucrose cushion, followed by CsCl isopycnic gradient (1.36 g/cm^3) ultracentrifugation. To verify the morphology of VLP using the NDV system, the ORF2 of NV was cloned into bacmids and transfected into *Spodoptera frugiperda* (Sf9) cells (Invitrogen). Baculovirus-expressed VLPs were purified and examined by EM. VLPs of approximately 35–40 nm in diameter, similar to the size of baculovirus-expressed VLPs were observed in embryonated egg preparations, indicating self-assembly of VP1 protein produced by the two rNDV vectors into VLPs (Fig. 2B).

Induction of NV-specific immune responses in mice

Immunogenicity of rNDV vectors containing ORF2+3+3′UTR was evaluated in BALB/c female mice (5 mice per group). Mice

were immunized individually with virus (30 μl of each, 10^6 EID_{50}) by the intranasal route after inhalational anesthesia. For comparison, two groups of mice were immunized with rLaSota-NV ORF2 and 30 μg of baculovirus-expressed VLPs (Fang et al., 2013). Mice received three doses of immunization with two-week intervals. The last group of mice was inoculated with PBS as an unvaccinated control. All infected mice had no apparent clinical signs during the immunization.

Serum samples were collected from pre-immunized mice and immunized mice at 5 and 6 week postinoculation (wpi). First, antibody-mediated neutralization for NV was determined by using a histoblood group antigen (HBGA) binding blocking assay (Reeck et al., 2010; Lindesmith et al., 2012). We confirmed the blockade ability of sera from mice immunized with vaccine viruses to block NV VLP-carbohydrate interactions (Fig. 3A). Both rBCm-NV ORF2+3+3′UTR and rLaSota-NV ORF2+3+3′UTR showed similar levels of blockade ability to that of baculovirus-expressed VLPs in mice ($p > 0.05$). NV-specific antibody response in mice was evaluated by determining the serum IgG antibody by ELISA (Fig. 3B). At 5 wpi, rBCm-NV ORF2+3+3′UTR, rLaSota-NV ORF2+3+3′UTR and baculovirus-expressed VLPs induced similar levels of NV specific IgG response in mice ($p > 0.05$). At 6 wpi, rBCm-NV ORF2+3+3′UTR and baculovirus-expressed VLPs induced higher levels of IgG response than rLaSota-NV ORF2 and rLaSota-NV ORF2+3+3′UTR in immunized mice ($p < 0.05$). Therefore, our results suggest that rBCm-NV ORF2+3+3′UTR induced similar levels of IgG response compared to that induced by purified baculovirus-expressed VLPs in mice.

The systemic NV-specific IgG response was further characterized into Th1 and Th2 responses by measuring IgG antibody subtypes IgG2a and IgG1, respectively (Fig. 3C and D). The baculovirus-expressed VLPs induced the highest level of IgG1 subtype ($p < 0.05$), resulting in a Th2/Th1 ratio of 2.5. In contrast, both rLaSota and rBCm containing ORF2+3+3′UTR induced high levels of IgG2a response compared to rLaSota-NV ORF2 and baculovirus-expressed VLPs ($p < 0.05$), resulting in a Th1/Th2 ratio of 3.5 and 6.3, respectively. This result suggests a difference in induction of systemic NV-specific immune response between live NDV vaccines and nonreplicating baculovirus VLPs.

This was further evaluated by measuring specific cellular immune response from mouse splenocytes (Fig. 3E). At 6 wpi, the spleens were collected from sacrificed mice for detection of IFN-γ, TNF-α, and IL-2 levels by a cytokine specific enzyme-linked immunospot (ELISPOT) assay. Among the immunized groups, rBCm-NV ORF2+3+3′UTR produced the highest levels of IFN-γ, IL-2, and TNF-α ($p < 0.005$). In contrast, rLaSota-NV ORF2 and baculovirus-expressed VLPs induced low levels of the three cytokines compared to rBCm-NV ORF2+3+3′UTR ($p < 0.05$).

Mucosal immune response in mice was evaluated by determining IgA titers by ELISA in the collected fecal samples at 5 and 6 wpi (Fig. 3F). The level of IgA was calculated from a standard curve that was determined by the absorbance values of the mouse IgA standard. rBCm-NV ORF2+3+3′UTR induced significantly higher levels of IgA responses than those induced by baculovirus-expressed VLPs and other rNDVs ($p < 0.05$). Baculovirus-expressed VLPs induced higher IgA response than rLaSota-NV ORF2 and rLaSota-NV ORF2+3+3′UTR ($p < 0.05$).

Discussion

NDV has shown promising results as a potential vaccine vector for human use (Bukreyev and Collins, 2008). We show here that the NDV vector can provide an alternative live vaccine platform for noroviruses and other non-cultivable pathogens of humans. Although both lentogenic and mesogenic NDV strains can be used as vaccine vectors, the mesogenic strains are more easily grown in vitro and are more immunogenic in vivo (Bukreyev et al., 2005). In our previous study, we expressed the VP1 protein of human norovirus (GII.4) using conventional rLaSota and rBCm vectors and showed that rBCm vector was more efficient than the rLaSota vector in inducing humoral, cellular and mucosal immune in mice (Kim et al., 2014). This indicated that rBCm has the potential to be a vaccine vector against norovirus infection in humans.

Previously, the expression of VP1 by baculovirus in insect cells was enhanced by the presence of the ORF3 and NV 3′UTR (Bertolotti-Ciarlet et al., 2003). These sequences located at the 3′ end of the NV genome contain cis-acting regulatory elements that are required for gene expression and translational regulation. Similarly, in this study, insertion of only ORF2 into rLaSota and rBCm vectors resulted in weak levels of expression of VP1 protein in DF1 cells and in allantoic fluid of embryonated chicken eggs. However, inclusion of ORF3 and 3′UTR in our NDV constructs enhanced the level of VP1 expression in DF1 cell lysates, in cell culture medium, and in allantoic fluid of embryonated chicken eggs. Furthermore, morphological analysis of VLPs produced by the two rNDV vectors showed similarity to those produced by the baculovirus expression system. Our study suggests that rNDV vector can produce large quantities of VLPs in embryonated chicken eggs at 3 dpi compared to baculovirus-expressed VLPs in insect cells at 7 dpi. Therefore, NDV vector can be a cost-effective, efficient, time-saving, and feasible approach for large-scale manufacture of various genotypes of norovirus VLP vaccines.

NV VLP vaccines have been immunogenic in animal models via parenteral, oral, or intranasal route (Guerrero et al., 2001). A NV VLP vaccine was evaluated in a phase II human trial, showing that a two-dose, intranasally administered NV VLP vaccine (100 μg) with adjuvants provided homologous protection against NV-associated viral gastroenteritis (Atmar et al., 2011). Human trial study suggests that the induction of IFN-γ and IgG2a antibody levels by VLP immunization can be important for efficient prevention of norovirus infection. Although the early norovirus vaccine trials appear promising (Ramani et al., 2014), there also exist several challenges to vaccine, such as large-scale manufacture of VLP vaccines and their protective efficacy against a broad range of norovirus strains. In general, the magnitude of the immune response to live viral vaccines over subunit protein or inactivated virus vaccines is substantially greater and broader in primary immunization (Belshe et al., 2007). Our previous study also supports the advantage of vaccination with live rNDVs expressing norovirus VP1 proteins over nonreplicating VLPs (Kim et al., 2014). In this study, optimization of VP1 protein expression by NDV was crucial to enhance the immunogenicity of rNDV-vectored vaccine for norovirus. As observed in our mouse study, expression of ORF2 in the presence of ORF3 and 3′UTR induced higher levels of immune response than that of ORF2 alone in the transcription unit, indicating that the amount of VP1 expression greatly affected the immune response in mice. VP2 might play a role in stabilization of VLPs (Bertolotti-Ciarlet et al., 2003). Although both rNDV vectors induced similar levels of IgG immune response, rBCm-NV ORF2+3+3′UTR induced higher levels of IgA and specific cellular immune responses than rLaSota-NV ORF2+3+3′UTR in mice, indicating that the NDV vector backbone can play an important role in induction of immune response. Our studies have also demonstrated that the expression of norovirus capsid proteins, GI and GII.4 did not increase NDV virulence for chickens; thus, showing the potential of a safe and effective vaccine for control of norovirus infection. In future studies, we will explore the protective efficacy of rNDV vectored vaccines expressing genotype GI and GII.4 VP1 proteins. It may be possible to formulate a bivalent vaccine for broad protection against norovirus infection.

Materials and methods

Generation of rNDVs expressing NV VP1 protein

The position between the P and M genes in the rNDV genome has been identified as the optimal location for expression of foreign genes without affecting replication of rNDV (Bukreyev and Collins, 2008). Therefore, the ORF2 (1593 nt) of Norovirus strain Norwalk virus (Hu/NV/Norwalk virus/1968/US) (Fernandez-Vega et al., 2004) was inserted into the intergenic region between the P and M genes in antigenomic cDNAs of NDV strain rLaSota and a modified strain rBC (Fig. 1). In addition, an expression cassette containing NV ORF2, ORF3, and 3′UTR (2297 nt) was placed into cDNA of rLaSota and rBCm vectors as described above. rNDVs were recovered using our standard procedure (Huang et al., 2001). The recovered recombinant viruses were passaged five times in embryonated chicken eggs and confirmed by RT-PCR and sequence analysis.

In vitro characterization of rNDVs expressing NV VP1 protein

Expression of the NV VP1 protein in DF1 cells and in embryonated chicken eggs was analyzed by Western blot using a monoclonal antibody to VP1. The ability of rNDV vectors to produce VLPs in embryonated eggs was evaluated by purification of the VLPs by CsCl isopycnic gradient (1.36 g/cm^3) ultracentrifugation and by negative-staining EM analysis (Vongpunsawad et al., 2013). To verify the morphology of VLP produced using the NDV system, the ORF2 of NV (Hu/NV/Norwalk virus/1968/US) was cloned into bacmids and transfected into *S. frugiperda* (Sf9) cells (Invitrogen). Baculovirus-expressed VLPs were purified and compared with VLPs produced by the NDV system.

The multicycle growth kinetics of rNDVs was evaluated in DF1 cells in the presence of 10% chicken egg allantoic fluid. Pathogenicity of rNDVs expressing NV VP1 protein was determined by the MDT in 9-day-old specific pathogen free (SPF) embryonated chicken eggs and by the ICPI test in 1-day-old SPF chicks (Alexander, 1989).

Immunogenicity of rNDVs expressing NV VP1 protein in mice

Groups of four-week-old female BALB/c mice (5 mice per group) were immunized with rLaSota-NV ORF2, rLaSota-NV ORF2+3+3′UTR, or rBCm-NV ORF2+3+3′UTR (30 μl of each, 10^6 EID_{50}) by the intranasal route after inhalational anesthesia. One group of mice was intranasally inoculated with 30 μg of baculovirus-expressed VLPs (Fang et al., 2013). Mice received three doses of immunization with two-week intervals. The last group of mice was inoculated with PBS as unvaccinated controls. Serum and fecal samples were collected from pre-immunized mice and immunized mice at 5 and 6 week postinoculation (wpi). To evaluate antibody-mediated neutralization, HBGA binding blocking assay was conducted by measuring the ability of serum antibodies to inhibit NV VLP binding to H type 1 and Lewis b antigens (Reeck et al., 2010; Lindesmith et al., 2012). VLP binding to carbohydrates in the absence of a serum sample was used as a positive control. For each sample, the 50% blocking titer (BT_{50}) was defined as the titer at which the OD reading (after subtraction of the blank) was 50% of the OD of the positive control. NV-specific IgG, IgG1, IgG2a, and IgA titers in serum were measured by an enzyme-linked immunosorbent assay (ELISA) (Ball et al., 1998; Fang et al., 2013). At 6 wpi, the spleens were collected from sacrificed mice for detection of IFN-γ, TNF-α, and IL-2 levels by a cytokine specific enzyme-linked immunospot (ELISPOT) assay (Lindesmith et al., 2005). The results are expressed as mean spot-forming cells (SFC) per 10^6 splenocytes of duplicate wells. Stool samples were analyzed for NV-specific and total IgA by ELISA (Ball et al., 1998). The level of IgA was calculated from a standard curve that was determined by the absorbance values of the mouse IgA standard. Fecal IgA responses were expressed as a ratio of the NV-specific IgA (ng/ml) to total IgA (μg/ml). Statistically significant differences in immune responses between immunized mouse groups ($p < 0.05$) were evaluated by one-way analysis of variance (ANOVA).

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